

AMENDMENTS TO THE SPECIFICATION

The Sequence Listing has been amended as requested by the Examiner to add certain omitted sequences as SEQ ID NOS. 24-29.

Please insert the attached amended Sequence Listing after the References section and before the Claims section of the specification and renumber the pages accordingly.

Please replace the indicated paragraphs with the following paragraphs to add the Sequence Listing identifiers to the specification:

On Page 13, starting at line 4

Preferably, the polypeptide will comprise amino acid sequence RSKAKWQTGTNPLYR (SEQ ID NO. 2), more preferably RSKAKNPLYR (SEQ ID NO. 3), or one or both amino acid sequences RSKAK (SEQ ID NO. 4) and NPLYR (SEQ ID NO. 5). Most preferably, the polypeptide will be a fragment of an integrin subunit.

Page 19, starting at line 11

Preferably, a polypeptide or fragment of the invention consisting of the binding domain of the integrin or core amino acid sequence of the binding domain or a homolog, analog or variant of the polypeptide or fragment is used in the testing or assaying for whether an agent is capable of binding to the binding domain of the integrin. Most preferably, the polypeptide or fragment will consist of the amino acid sequence

RSKAKWQTGTNPLYR (SEQ ID NO. 2) or RSKAKNPLYR (SEQ ID NO. 3).

Page 31, starting at line 17

Figure 16: Shows the amino acid sequence of the cytoplasmic domain of the $\beta 6$ subunit (SEQ ID NO. 9) as well as the amino acid sequences for the $\beta 1$ to $\beta 3$ subunits (SEQ ID NOS. 6-8), respectively;

Page 32, starting at line 9

Figure 20: Graph showing binding of ERK2 (GST.ERK2) to a 15 mer fragment of the $\beta 6$ cytoplasmic domain and which has the amino acid sequence RSKAKWQTGTNPLYR (SEQ ID NO. 2); and

Figure 21: Shows regions of the cytoplasmic domain of the $\beta 6$ subunit (SEQ ID NO. 10) corresponding to synthesised fragments thereof evaluated for capacity to be bound by ERK2.

Page 32, starting at line 14

Figure 23: Graph showing binding of ERK2 (thrombin cleaved) to synthesised peptide having the amino acid sequence RSKAKNPLYR (SEQ ID NO. 3) compared to the 15 mer RSKAKWQTGTNPLYR (SEQ ID NO. 2) fragment of the cytoplasmic domain of the $\beta 6$ subunit.

Figure 24: Graph showing binding of JNK-1 to the $\beta 6$ cytoplasmic domain.

Figure 25: location of $\beta 6$ $\Delta 746-764$ (SEQ ID NO. 19), $\beta 6(770t)$ (SEQ ID NO. 28) and $\beta 6(777t)$ (SEQ ID NO. 29) deletions in the cytoplasmic domain of the $\beta 6$ subunit (SEQ ID NO. 10).

On page 33, starting at line 5

Figure 28: Proliferation of HT29 colon cancer cells cultured for 48 hours and treated with penetratin, the fragment of $\beta 6$ cytoplasmic domain having amino acid sequence RSKAKWQTGTNPLYR (SEQ ID NO. 2) alone or the fragment coupled to penetratin for the final 24 hours of the incubation period.

Figure 29: Proliferation of SW480 cells expressing wild-type $\beta 6$ cultured on plastic for 48 hours and treated with penetratin, the RSKAKWQTGTNPLYR (SEQ ID NO. 2) peptide alone or the peptide coupled to penetratin for the final 24 hours of the incubation period.

Figures 30 (A) to 29 (C): (A) SW480 cells cultured with control additive for 24 hours; (B) SW480 cells cultured with penetratin for 24 hours; (C) SW480 cells cultured with RSKAKWQTGTNPLYR (SEQ ID NO. 2) bound to penetratin for 24 hours.

Figures 31(A) and 31(B): (A) SW480 mock ($-\beta 6$) and SW480 $\beta 6$ transfectants ($+\beta 6$) cultured in presence of the RSKAKWQTGTNPLYR (SEQ ID NO. 2) peptide coupled to penetratin. (B) Photomicrographs of cells shown in (A) cultured in the

presence/absence of the peptide penetratin complex.

On page 34, starting at line 1

Figures 33(A) and 33(B): Graphs showing inhibition of proliferation of SW480 cells expressing full length wild-type $\beta 6$ in the presence of RSKAKWQTGTNPLYR (SEQ ID NO. 2) peptide bound to penetratin.

Figure 34: Graph showing binding of ERK2 to RSKAKWQTGTNPLYR (SEQ ID NO. 2) peptide and peptides corresponding to regions of the cytoplasmic domain of $\beta 3$ and $\beta 5$.

On page 49, starting at line 16

Polypeptides including fusion proteins and fragments of an integrin subunit comprising the binding domain for a MAP kinase or incorporating sufficient core amino acid sequence of the binding domain for binding by the MAP kinase are encompassed by the present invention. Typically, a polypeptide of the invention will have a length of about 150 amino acids or less, more preferably about 100 or 50 amino acids or less and generally, less than about 40 amino acids. Preferably, the length will be from between about 5 to about 30 amino acids, and more preferably from between about 5 amino acids and about 25 amino acids. Preferably, a polypeptide will comprise or incorporate the amino acid sequence RSKAKWQTGTNPLYR (SEQ ID NO. 2), more usually the

amino acid sequence RSKAKNPLYR (SEQ ID NO. 3), or one or both of sequences RSKAK (SEQ ID NO. 4) and NPLYR (SEQ ID NO. 5).

On page 55, starting at line 8

Specific targetting to $\beta 6$ -expressing cancer cells may also be achieved by coupling humanised anti- $\beta 6$ antibody to carrier molecules such as penetratin coupled to an agent capable of inhibiting binding of a MAP kinase with an integrin expressed by the cell or down regulation of the expression of the integrin. Coupling may for instance be by a peptide bond or disulfide bridge. Given that $\beta 6$ expression enhances effective proteolysis at the cell surface by matrix metalloproteinase-9 (Agrez et al, 1999), such targetting approaches may include engineering an MMP-9 cleavage site between the antibody and the carrier peptide penetratin to facilitate internalisation of the penetratin-agent complex. Another approach may employ coupling the penetratin-agent complex to $\beta 6$ integrin receptor-targetted peptides, targetted for binding to the extracellular $\beta 6$ domain by virtue of their DLXXL sequence. For example, a ligand recognition motif for $\alpha V\beta 6$ integrin, RTDLDSLRTYTL (SEQ ID NO. 24) (Kraft et al, 1999) may be used in conjunction with or without an engineered MMP-9 cleavage site to release the penetratin-agent complex at the cell surface. Further protocol for targetting nucleic acids

to cells by targetting integrins is described in Bachmann et al, 1998.

On page 68, starting at line 8

mRNA levels for $\beta 6$ expression were evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from cell cultures using the commercial TriPure isolation reagent based on the method of Chomczynski and Sacchi (1987). 0.4-2 μ g of RNA was used to prepared cDNA by reverse transcription. Briefly, a reaction mixture in a final volume of 40 μ l containing 8 μ l of 5 x RT reaction buffer (250mM Tris, 15mM MgCl₂ , 375mM KCL, pH 8.3), 8 μ l of 2.5mM of each dNTP, 4 μ l of 100mM DTT, 40U of an Rnase inhibitor, Rnasin (Promega, Madison, Wisconsin, USA), 0.5 μ g of random hexamers (Promega) and 200U of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega) were mixed and incubated at 38°C for a minimum of 90min. The reaction was stopped by heating at 95°C for 5 min and cDNA stored at 4°C until PCR. 2-5 μ l of this cDNA was combined with 5 μ l of 10 x PCR buffer (100mM Tris, 500mM KCl, 15mM MgCl₂ , pH8.3) 8 μ l of 1.25mM dNTP each and 1.25 μ l of 20 μ M of both forward and reverse primers. The forward primer sequence was 5'AGGATAGTTCTGTTTCCTGC3' (SEQ ID NO. 25) and the reverse primer

sequence 5'ATCATAGGAATATTTGGAGG3' (SEQ ID NO. 26). The reaction was initiated by 2.5U of Taq polymerase in a final volume of 50 μ l. After an initial 5 min incubation at 94°C, 30 cycles of amplification were performed under the following conditions: 94°C 1 min, 54°C 1 min and 72°C for 1 min. The reaction was stopped by incubating at 72°C for 10 min. To verify that equal amounts of RT product from cells were subjected to PCR amplification, the same amounts of cDNA were amplified for the "house-keeping" gene GAPDH using specific primers. The same reaction conditions were used except that the annealing temperature was changed to 48°C and PCR amplification performed for 35 cycles.

On page 84, starting at line 19

The region of the β 6 tail (SEQ ID NO. 9) to which each corresponds is indicated in Fig. 16 and set out below.

Fragment 1: HDRKEVAKFEAERSKAKWQTGT (SEQ ID NO. 11)

Fragment 2: RSKAKWQTGTNPLYRGSTST (SEQ ID NO. 12)

Fragment 3: NPLYRGSTSTFKNVTYKHRE (SEQ ID NO. 13)

Fragment 4: FKNVTYKHREKQKVDLSTDS (SEQ ID NO. 14)

On page 86, starting at line 11

To further localise the binding domain on the cytoplasmic tail of the β 6 subunit, progressively shorter peptides from the

region of the $\beta 6$ cytoplasmic tail corresponding to peptide fragment 2 were synthesised, biotinylated and the capacity to associate or otherwise bind to ERK2 assessed as described above. The binding of GST.ERK2 to a 15 mer test peptide (seq. 4) (SEQ ID NO. 2) having the amino acid sequence RSKAKWQTGTNPLYR (SEQ ID NO. 2) and a 10 mer test peptide having the sequence RSKAKWQTGT (SEQ ID NO. 15) is shown in Fig. 20 compared to fragment 2 over a range of concentrations of the peptides. As can be seen, no reduction in binding to the seq. 4 (SEQ ID NO. 2) peptide compared to fragment 2 was found. Binding of ERK2 to the seq. 3 peptide was substantially less than that observed for seq. 4 (SEQ ID NO. 2).

A number of 10 mer biotinylated peptides corresponding to regions of fragment 2 or fragment 3 were then tested. The amino acid sequence for each peptide is as follows and their location in the $\beta 6$ cytoplasmic domain (SEQ ID NO. 10) is indicated in Fig. 21.

10(1):NPLYRGSTST (SEQ ID NO. 16)

10(2):WQTGTNPLYR (SEQ ID NO. 17)

10(3):KFEAERSKAK (SEQ ID NO. 18)

The results are set out in Fig. 22 and show that GST.ERK binding to the 10 mer peptides is substantially reduced compared to binding to the seq. 4 (SEQ ID NO. 2) peptide suggesting that opposite end regions of seq. 4 (SEQ ID NO. 2) participate in the

binding of ERK2.

Comparable binding of ERK2 to seq. 4 was found using a further 10 mer peptide identified as 10(4) (SEQ ID NO. 3) in which amino acid sequence WQTGT (SEQ ID NO. 27) of seq. 4 is omitted indicating that WQTGT is a linker sequence that does not participate directly in the binding of ERK to seq. 4 (SEQ ID NO. 2). Negligible binding of ERK2 to the 5 mer peptide RSKAK (SEQ ID NO. 4) was observed as shown in Fig. 23. ERK2 cleaved from GST-ERK2 by thrombin was used in this assay. Results (not shown) indicate that greater than a 3 fold increase in assay sensitivity can be achieved using thrombin cleaved ERK2 rather than GST-ERK2.

On page 88, starting at line 7

To examine the role of the amino acid sequence RSKAKWQTGTNPLYR (SEQ ID NO. 2) in the $\beta 6$ cytoplasmic domain *in situ*, a $\beta 6$ deletion construct lacking the coding sequence for AERSKAKWQTGTNPLYRG (SEQ ID NO. 19) was transfected into colon cancer cell line SW480 which does not constitutively express the $\alpha V\beta 6$ integrin using the calcium phosphate method previously described for transfections into this cell line (Agrez et al, 1994). The location of the $\beta 6$ $\Delta 746-764$ (SEQ ID NO. 19) deletion is indicated in Fig. 25. Construction of the $\beta 6$ $\Delta 746-764$ (SEQ ID NO. 19) deletion mutant in the vector pcDNA1neo and failure of

the expressed receptor to localise to focal adhesions in Chinese hamster ovary cells has been reported (Cone et al, 1994). Facscan analysis revealed comparable levels of surface expression of mutant $\beta 6$ to that seen for the full length wild-type receptor (see Fig. 26).

Equal protein loads of cell lysates prepared from SW480 cells were immunoprecipitated with either anti- $\beta 6$ monoclonal antibody (mAb R6G9) or matched isotype control antibody. Surface biotinylation prior to immunoprecipitation confirmed equal surface expression of mutant and wild-type $\beta 6$ (see Fig. 27 (A)). Aliquots of the immunoprecipitates were electrophoresed and transferred to nitrocellulose for Western blotting using monoclonal antibody E10 which recognises ERK1/2. As seen in Fig. 27(B), loss of the RSKAKWQTGTNPLYR (SEQ ID NO. 2) sequence in the $\beta 6$ cytoplasmic domain reduced levels of $\beta 6$ -bound ERK by greater than approximately 75% of that observed for the wild type receptor.

On page 89, starting at line 6

HT29 and SW480 $\beta 6$ -expressing colon cancer cell lines were seeded into wells of 96-well microtitre plates (Nunc) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, glutamine, Hepes, and antibiotics. Seeding

cell densities were 3×10^3 cells per triplicate well for each condition tested and after 24 hours incubation of cell cultures in 5% CO₂, 100% humidity at 37°C, the culture medium was exchanged for serum-free DMEM medium supplemented with insulin, transferrin, selenous acid, hydrocortisone, non-essential amino acids, glutamine, Hepes and antibiotics containing either peptide RSKAKWQTGTNPLYR (SEQ ID NO. 2) alone or penetratin-peptide complex at a concentration of 10 μ m for HT29 cells or 30 μ m for SW480 β 6-expressing cells. Cell cultures were incubated for a further 24 hours following which cultures were photographed (Kodak Techpan Film at 100 ASA setting) and the experiments terminated by addition of the cell proliferation reagent WST-1 (Boehringer Mannheim) to monitor effects of the peptide on cell growth. The cell proliferation reagent WST-1 is designed to be used for the non-radioactive, spectrophotometric quantification of cell growth and viability in proliferation and chemosensitivity assays. The colourmetric assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells.

On page 90, starting at line 15

SW480 mock and SW480 β 6 transfectants were cultured in DMEM medium supplemented with 1% foetal bovine serum in the presence of 20 μ M seq. 4 (SEQ ID NO. 2) coupled to penetratin. Percentage

inhibition was assessed by the WST-1 colorimetric dehydrogenase assay described in Example 6. The percentage inhibition of growth observed for the $-\beta 6$ and $+\beta 6$ expressing cells was 17% and 50%, respectively as indicated in Fig 31A. The $-\beta 6$ and $+\beta$ cultured cells are shown in Fig 31B.

On page 91, starting at line 16

Growth inhibition of SW480 cells expressing full length wild-type $\beta 6$ exposed to seq.4 coupled to penetratin or RSKAKWQTGTNPLYR (SEQ ID NO. 2) peptide coupled to penetratin (5, 10, 20, 30 μ M in DMEM minus foetal bovine serum) but which peptide contained alanine substitutions at the four positions indicated was assessed. As shown in Fig.33(A) and Fig 33(B), progressive inhibition of proliferation in a dose-response manner was observed for the seq.4 penetratin complex compared with the alanine substituted peptide-penetratin complex which was without effect at all doses tested.

On page 92, starting at line 3

Binding of ERK2 to the seq.4 peptide (RSKAKWQTGTNPLYR) (SEQ ID NO. 2) was compared with peptides corresponding to regions of the cytoplasmic domain of integrin subunits $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$. The amino acid sequences for those peptides is shown below:

Application No.: 10/019,816
Filed: March 27, 2003
TC Art Unit: 1644
Confirmation No.:9944

- β 1 KFEKEKMNAKWDGTGENPIYK (SEQ ID NO. 20)
- β 2 KEKLKSQWNNDNPLFK (SEQ ID NO. 21)
- β 3 RARAKWDTANNPLYK (SEQ ID NO. 22)
- β 5 RSRARYEMASNPLYR (SEQ ID NO. 23)

As shown in Fig.34, significant binding of ERK2 to the seq. 4 peptide (SEQ ID NO. 2) was observed. Binding of ERK2 to the β 5 and β 3 peptides was also found. The results have been corrected for non-specific binding and indicate a hierarchy of binding of ERK2 to integrin subunits.